UNIVERSITI PUTRA MALAYSIA

# CLONING AND EXPRESSION OF VP2 GENE OF CHICKEN ANEMIA VURUS STRAIN CUX-1 IN *LACTOCOCCUS LACTIS MG1363*

NURFILZA AHMAT

FSAS 2001 41

# CLONING AND EXPRESSION OF VP2 GENE OF CHICKEN ANEMIA VIRUS STRAIN CUX-1 IN *LACTOCOCCUS LACTIS* MG1363

**By**

**NURFILZA AHMAT**

**Thesis Submitted in Fulfilment of the Requirement for the Degree of Master of Science in the Faculty of Science and Environmental Studies Universiti Putra Malaysia**

**March 2001**

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science.

# CLONING AND EXPRESSION OF VP2 GENE OF CHICKEN ANEMIA VIRUS STRAIN CUX-1 IN *LACTOCOCCUS LACTIS* MG1363

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**March 2001**

**Chairperson:** **Abdullah B. Sipat, Ph.D.**

**Faculty:** **Faculty of Science and Environmental Studies**

In this study, the *lactococcal* plasmid vector pMG36e, was exploited for the cloning, replication and expression of a viral protein-2 (VP2) gene in the bacterial host, *Lactococcus lactis* strain MG1363. The VP2 gene (0.65 kb) of chicken anemia virus (CAV) of strain Cuxhaven-1 was amplified from viral genomic DNA by polymerase chain reaction (PCR) using the VP2FX and VP2RSal primers (Table 6), cloned into the expression vector pMG36e, and electrotransformed into the *L. lactis* MG1363 host. Sequencing of the recombinant plasmid pMG36e-VP2 using the Forward and Reverse primers (Table 6), showed 99% homology of the VP2 insert with that of the published VP2 CAV Cux-1 sequence. SDS-PAGE and Western blot hybridization of *L. lactis* (pMG36e-VP2; 4.3 kb), showed no significant protein band of VP2 gene product (24 kDa). However, the gene was transcribed under the P32 promoter of the expression vector pMG36e as shown by a 0.7 kb reverse-transcribed product of the VP2 gene.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains.

**PENGKLONAN DAN PENZAHIRAN GEN VP2 VIRUS ANEMIA AYAM STRAIN CUX-1 DI DALAM *LACTOCOCCUS LACTIS* MG1363**

Oleh

**NURFILZA AHMAT**

**Mac 2000**

**Pengerusi: Abdullah B. Sipat**

**Fakulti: Sains dan Alam Sekitar**

Di dalam penyelidikan ini, vektor penzahir *Lactococcus*, pMG36e, telah digunakan untuk mengklon, mereplikasi serta menzahirkan protein virus 2 (VP2), di dalam bakteria perumah *Lactococcus lactis*. Gen VP2 (0.65 kb) virus anemia ayam dari strain Cuxhaven-1 telah digandakan dari DNA genom virus melalui kaedah reaksi berantai polimeres (PCR) dengan menggunakan primer-primer VP2FX dan VP2RSal (Jadual 6), diklon ke dalam vektor penzahir pMG36e, dan akhirnya dimasukkan ke dalam sel perumah *L. lactis* MG1363. Penjujukan plasmid rekombinan pMG36e-VP2 tersebut menggunakan primer-primer Forward dan Reverse (Jadual 6) menunjukkan 99 % persamaan di antara jujukan gen selitan VP2 dengan jujukan gen VP2 Cux-1 yang telah diterbitkan. Kaedah SDS-PAGE dan penghibridan Western tidak berjaya mengesan protin dari gen VP2 (24 kDa). Walau bagaimanapun, proses transkripsi telah berlaku di bawah promoter P32 pada vektor penzahiran pMG36e, yang ditunjukkan dengan penghasilan produk 'reverse-transcribed' VP2 bersaiz 0.7 kb.

# ACKNOWLEDGEMENTS

*Alhamdulillah, praise be to Allah*

The author would like to thank everyone especially the supervisory committee, colleagues and friends for their invaluable guide and help throughout the long course of the project.

The author also wishes to express her thanks to her family and personal friends for their moral support and encouragement. Their prayers and good wishes have helped her through the difficult and trying times.

*"As an adolescent, I aspired to lasting fame, I craved factual certainty, and I thirsted for a meaningful vision of human life -- so I became a scientist. This is like becoming an archbishop so you can meet girls." M. Cartmill.*

I certify that an Examination Committee met on 19th March 2001 to conduct the final examination of Nurfilza Ahmat, on her Master of Science thesis entitled "Cloning and Expression of VP2 Gene of Chicken Anemia Virus Strain Cux-1 in *Lactococcus lactis* MG1363" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

TAN SIANG HEE, PhD.
Institute of Bioscience
Universiti Putra Malaysia
(Chairman)

ABDULLAH SIPAT, Ph.D.
Associate Professor
Faculty of Science and Environmental Studies
Universiti Putra Malaysia
(Member)

KHATIJAH MOHD. YUSOFF, Ph.D.
Associate Professor
Faculty of Science and Environmental Studies
Universiti Putra Malaysia
(Member)

RAHA ABDUL RAHIM, Ph.D.
Faculty of Food Science and Biotechnology
Universiti Putra Malaysia
(Member)

MOHD. GHAZALI MOHAYIDIN, Ph.D.
Professor/Deputy Dean of Graduate School,
Universiti Putra Malaysia

Date: 17 MAY 2001

This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Master of Science.

AINI IDERIS, Ph.D.
Professor/Deputy Dean of Graduate School,
Universiti Putra Malaysia

Date: 14 JUN 2001

## DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

NURFILZA AHMAT

Date: 17th May, 2001

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# LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| AP | - | alkaline phosphatase |
| bp | - | base pair |
| CAV | - | chicken anemia virus |
| cat | - | chloramphenicol acetyl transferase |
| $Cm^R$ | - | chloramphenicol resistance |
| DEPC | - | diethyl pyrocarbonate |
| DIG | - | digoxigenin |
| DNA | - | deoxyribonucleic acid |
| ds | - | double stranded |
| EDTA | - | ethylenediamine tetraacetate |
| ELISA | - | enzyme-linked immunosorbant assay |
| Em | - | erythromycin |
| $Em^R$ | - | erythromycin resistance |
| EtBr | - | ethidium bromide |
| kb | - | kilo base pair |
| kDa | - | kilo Daltons |
| LB | - | Luria Bertani |
| M | - | Molarity |
| MCS | - | multiple cloning site |
| MDCC-MSB1 | - | Marek's disease virus transformed lymphoblastoid cell line |
| OD | - | optical density |
| ORF | - | open reading frame |
| PCR | - | polymerase chain reaction |
| RBS | - | ribosomal binding site |
| RE | - | restriction enzyme |
| RF | - | replicative form |
| RNA | - | ribonucleic acid |
| rRNA | - | ribosomal RNA |
| mRNA | - | messenger RNA |
| SDS-PAGE | - | sodium dodecyl sulphate-polyacrylamide gel electrophoresis |
| SS | - | single-stranded |
| STE | - | sodium tris-EDTA |
| TAE | - | Tris-acetate-EDTA |
| TTFC | - | tetanus toxin fragment C |

# CHAPTER I

## INTRODUCTION

Chicken anemia virus (CAV) is an icosahedral, single-stranded DNA virus of genus circovirus in the family *Circoviridae*, which causes anemia in young susceptible chicks and subclinical infections in older chickens (McNulty *et al.*, 1991). CAV has three immunogenic major proteins. The viral protein 1 (VP1) has been shown to be the only component found in the capsid viral protein (Todd *et al.*, 1990; Noteborn *et al.*, 1992a). On the other hand, viral protein 2 (VP2) is suspected to be an early gene regulatory protein (Douglas *et al.*, 1995) or a scaffold protein that interacts transiently with VP1 (Koch *et al.*, 1995; Noteborn *et al.*, 1998). It has been suggested to have an important role in the formation of the CAV neutralizing epitope on VP1 (Koch *et al.*, 1995; Noteborn *et al.*, 1998). Meanwhile, viral protein 3 (VP3) has been identified as an apoptin, which causes apotosis in avian transformed lymphoblastoid infected cells as well as several human tumor cells (Noteborn *et al.*, 1994; Van Oorschot *et al.*, 1997). The only commercial vaccine available for CAV is the live unattenuated form of the virus (Vielitz and Landgraf, 1988, Koch *et al.*, 1995). Since the CAV infection is prevalent worldwide and has been cited to cause economic loss of up to 30 %, by causing direct mortality and subclinical.diseases as well as by aggravating other avian infections (McNulty *et al.*, 1991), it is imperative that a vaccine be developed. The interest of this particular work is to construct a recombinant vector system, using the bacterium *Lactococcus lactis*, which carries a CAV VP2 gene encoding for the VP2 protein, with the potential to function as a vaccine delivery vehicle.

Lactic acid bacteria (LAB) have long been noted for its food-grade status, having been widely used in the fermentation industry; however, its potential role as a vaccine delivery shuttle has been exploited only recently (de Vos *et al.*, 1997). One of the LAB genus, *Lactococcus*, in particular the *L. lactis* species has the advantage of being non-pathogenic, non-colonizing and non-invasive (Wells *et al.*, 1996), cheap to maintain (de Vos, 1986), having low innate immunogenicity (Norton *et al.*, 1994), and an already relatively extensive genetic background (de Vos *et al.*, 1989). It has been used for the development of promising hosts for the safe production of commercially important food-grade heterologous proteins for fermentative applications (Leenhouts and Venema, 1992). Quite a number of heterologous genes have been expressed by *L. lactis* and some are even secreted (Table 3). Recently, its use has extended to medical applications, i.e. as vaccine delivery vehicles (Leenhouts and Venema, 1992).

The problems for effective vaccines lie in their ability to induce protective antibodies, their safety, and the effectiveness of the vaccination administration techniques. Vaccine researchers are now challenged by the need to come up with those vaccines that will simplify vaccine distribution and administration. Moreover, it must also be safe, effective and capable of eliciting active immunity at early age, as well as reducing dependency on a cold chain, be suitable for oral administration, and be low in cost; all of which are qualities of good vaccines (Wells, *et al.*, 1996).

Most of the presently available vaccines are either killed or live attenuated vaccines, which carry risk factors to immunosuppressed or immunologically susceptible hosts. Since *Lactococcus* is a 'generally regarded as safe' (GRAS) host, it has been chosen as an alternative host due to its GRAS status, and its suitability as a mucosal route vaccine delivery vehicle, as well as all its advantages mentioned previously. The use of a *lactococcal* expression system to express the VP2 protein is a novel approach for a recombinant vaccine. However, for the same advantages mentioned, certain weaknesses should also be taken into account, such as its non-adhesive property, its capacity of inducing tolerance in hosts because of the food grade status, and its non-aggressive approach, which sometimes is synonymous to being ineffective or limited in application, to name just a few. Therefore, more in depth studies are needed before the system can be perfected.

## OBJECTIVES

1) To amplify the VP2 gene of the CAV Cux-1 genomic DNA by polymerase chain reaction.
2) To clone the VP2 PCR fragment in the *lactococcal* expression vector pMG36e. The resulting recombinant plasmid is to be transformed and maintained in *L. lactis* MG1363.
3) To characterize the recombinant plasmid.
4) To monitor the expression of the VP2 gene.

# CHAPTER II

# LITERATURE REVIEW

## Chicken Anemia Virus (CAV)

### Introduction

Yuasa *et al*. first discovered chicken anemia virus, or CAV, from commercial chickens during the investigation of a Marek's disease vaccine accident with reticuloendotheliosis virus (REV), which occurred in Japan in 1974, and it was finally described in 1979 (Yuasa *et al*., 1979). CAV is a small, non-enveloped, icosahedral virus (McNulty *et al*., 1990; Noteborn *et al*., 1991) with a diameter of about 23 nm (Gelderblom *et al*., 1989) to 23.5 nm (Todd *et al*., 1990). In cesium chloride gradients, CAV has a buoyant density of 1.33 to 1.34 g/ml. It is also highly resistant to environmental inactivation and some common disinfectants. Electron microscopic (Gelderblom *et al*., 1989; McNulty *et al*., 1990), biochemical (Todd *et al*., 1990), and DNA and RNA (Claessens *et al*., 1991; Noteborn *et al*., 1991; Phenix *et al*., 1994) studies have established that the agent is a virus with a negative, single-stranded, circular DNA genome of 2.3 kb. The genomic structure of CAV is similar to two other small viruses, porcine circovirus (Tischer *et al*., 1982) and psittacine beak and feather disease virus (Ritchie *et al*., 1989), which is the basis for the assignment of CAV to the circovirus genus under *Circoviridae* family (Fenners *et al*., 1993).

CAV prevalence is now reported in many countries and is believed to be a major causative agent of an anemic and immunosuppressive disease in chicken (McConnell *et al.*, 1993). Diagnosis of sub clinical CAV infections is often ambiguous since flock history, clinical signs, and gross pathology are generally inconclusive. Virus isolation, either in one-day-old chicks or in cell cultures such as MSB1, is necessary for a definitive diagnosis but it is time-consuming (Gelderblom *et al.*, 1989), moreover, some CAV strains have failed to replicate in MSB1 or other lymphoblastoid cell lines (Renshaw *et al.*, 1996). A universal molecular diagnostic approach such as using PCR or PCR ELISA has yet to be fully developed for CAV diagnosis.

Prevention and control of the disease is important to curb it from rendering a further economic loss to farmers and their countries. Other than stress-free management of broiler breeders to prevent uncontrolled spread of the disease, vaccination is an obvious alternative. The only vaccine available now is the unattenuated live virus version (Vielitz and Landgraf, 1988); researchers are now working on live attenuated and killed inactivated vaccines in an effort to control the disease (Fussel, 1998; Rosenberger and Cloud, 1998). This study, on the other hand, is looking to provide a solution in the form of a recombinant vaccine vector for CAV, using a safe genre of bacteria, i.e. *Lactococcus lactis*.

### *Circoviridae* and Circoviruses

The virus family *Circoviridae*, characterized by single stranded DNA viruses commonly found in vertebrate and plant hosts, is comprised of the genus circovirus (Lukert *et al.*,

1995). The genus circovirus has tentatively listed three species members, namely, chicken anemia virus (CAV), beak and feather disease virus (BFDV), and porcine circovirus (PCV), which comprise the animal circoviruses (Lukert *et al*., 1995). There are also unassigned viruses in the family, which include the banana bunchy top virus (BBTV), coconut foliar decay virus (CFDV), and subterranean clover stunt virus (SCSV), all of which are referred to as plant circoviruses (Lukert *et al*., 1995). Recently, a new circovirus, the TT virus (TTV) was discovered and described as the first human circovirus (Nishizawa *et al*., 1997; Okamoto *et al*., 1998; Miyata *et al*., 1999).

Lately, however, arguments have arisen disputing the appropriateness of placing CAV in the *Circoviridae* family (Bassami *et al*., 1998). It has been shown from previous work and studies that CAV is morphologically, genomically, and antigenically different from the other circoviruses (Todd *et al*., 1991, Bassami *et al*., 1998), and it remains to be decided whether CAV should be classified together with these other viruses.

**Propagation of CAV in Cell Lines**

CAV can be grown in a Marek's disease virus-transfected chicken lymphoblastoid (MDCC-MSB1; Akiyama and Kato, 1974) cell line or B cell line 1104-X-5 from chickens with avian lymphoid leucosis (Hihara et al., 1974). The MDCC-MSB1 cells can be further divided into two sublines, MSB1 (L), e.g. isolate CU147, and MSB1 (S), e.g. isolate CU205 (Schat *et al*., 1992). Some CAV strains, however, have failed to replicate in MSB1 or other lymphoblastoid cell lines. For example, in a study by Renshaw *et al*. (1996), it was discovered that the CAV isolates CIA-1 (Lucio *et al*.,

1990) and L-028, both isolated in the United States, were incapable of infecting the normal MSB1 (L) cells completely, but were able to infect MSB1 (S) with a significantly slower spread rate when compared with Cux-1. The decreased rate of spread was shown to be associated with 1 or 2 specific amino acid changes in a hyper variable region present in the VP1 region. On the other hand, the complete block of infection by CIA-1 in MSB1 (L) cells might be due to 5 amino acid changes in a region upstream from the hyper variable region causing a change in the conformational interaction between VP1 and VP2 (Renshaw *et al*., 1996).

The existence of CAV strain-specific sublines suggested underlying biological differences between CAV isolates which disabled them from infecting normally CAV permissive cell lines. Furthermore, Renshaw *et al.* (1996) also reported the appearance of CAV-resistant subpopulations of cells, shown by the fluctuation of percentages of antigen positive cells, which suggested that both cell and virus populations predominate at different times. A further study on the variation among CAV isolates may be able to spot differences in pathogenic potential and be useful in the development of vaccine for protection against a range of isolates (Renshaw *et al*., 1996). On the other hand, Noteborn *et al.* (1991), who reported that 1104-X-5 cells are less sensitive to CAV infections when compared to MSB1 cells, suggested that the absence of a certain enhancer element, that is one of the five tandem repeats in the non-coding region of CAV genome, might help explain the phenomenon. Each repeat has a sequence homologous to that, which binds adenovirus transcription factor (ATF) site, and acts a transcription stimulator. The absence of one repeat could affect transcription as a whole,

and therefore, the infective process. A further study into this claim might also give a deeper insight to the factors, which contribute to the virulence of CAV.

**Molecular and Genomic Properties**

Gelderblom *et al.* (1989) and Todd *et al.* (1990) have demonstrated circular single-stranded CAV DNA molecules of length between 2,298 (Meehan *et al.*, 1992) and 2319 (Noteborn *et al.*, 1991) nucleotides. The CAV genome, which exists in the negative strand in virions (Phenix *et al.*, 1994), comprises three partially or completely overlapping open reading frames or ORFs (Fig. 1) for three viral proteins (VP's). The three genes, VP1 (1.35 kb), VP2 (0.65 kb), and VP3 (0.34 kb) encode for proteins of 52, 24 and 13 kDa, which have been designated proteins VP1, VP2 and VP3 respectively (Noteborn *et al.*, 1994).

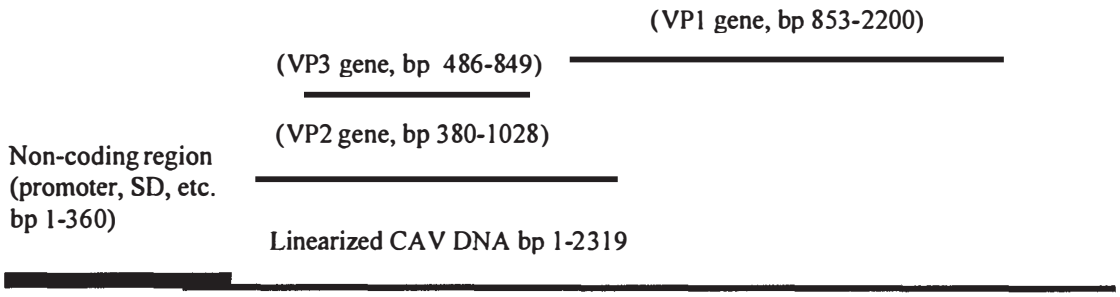


Figure 1: A schematic diagram of a linearized Cux-1 CAV genomic DNA showing the partial overlapping of its three major proteins.

In the non-coding region of the CAV genome, upstream from the ORFs , there is one apparent promoter at position 323-329, and one poly (A) addition signal downstream from the ORFs at bp 2287-2302, suggesting that CAV may encode a polycistronic

mRNA. One unique feature of the CAV genome is the presence of an array of four or five of 19 or 21 bps perfect and near perfect tandem repeats situated between bp positions 144 and 260 with a gap of 12 bps between repeat units 3 and 4, believed to possess regulatory sequences for gene expression (Noteborn *et al.*, 1991; Meehan *et al.*, 1992, Miyata *et al.*, 1999). Each repeat contains a sequence that is homologous to that which binds the adenovirus transcription factor (ATF) site (Noteborn *et al.*, 1991) and that which binds the cyclic AMP responsive element binding protein (CREB) site (Noteborn *et al.*, 1991, Meehan *et al.*, 1992). In addition, polypurine and polypyrimidine tracts with possible significance as chicken SP 1 binding sites are also present (Meehan *et al.*, 1992). Sequences that may be recognized by the lymphoid-specific NF-κβ factor are also present, which might have a role in the high cell-specificity exhibited by CAV (Meehan *et al.*, 1992). There is also a protected DNA region of length 90 to 100 bp most probably due to a GC-rich region around position 2245, which is predicted to form a stable hairpin structure (Noteborn *et al.*, 1992a). The genomic organization of CAV differs slightly in different isolates; Table 1 shows the genomic organizations of Cux-1 CAV, of length 2319 bp based on sequences in the database with accession number M55918 (Noteborn *et al.*, 1991).

Table 1: Genomic organization of Cux-1 CAV (adapted from Miyata *et al.*, 1999)

| Consensus Sequence | Position (length) in CAV |
|---|---|
| 36-nt stretch | 2237-2272 |
| GC-rich region | 2206-2272 |
| ORFs: | |
| VP1 (frame 1) | 853-2199 (1347 bp, 449 aa) |
| VP2 (frame 2) | 380-1027 (648 bp, 216 aa) |
| VP3 (frame 3) | 486-848 (363 bp, 121 aa) |

| | |
|---|---|
| SP-1 site (GGGCGG) | 305-310 |
| TATA (TATATA) | 323-329 |
| Poly (A) (AATAAA) | 2287-2302 |
| AP-2 sites (CCC[AC]N[CG][CG][CG]) | 2148-2155, 2222-2229, 2237-2244, 2241-2248 |
| ATF / CREB (ACGTCA) | 157-162, 178-183, 199-204, 232-237, 253-258 |
| NF- κβ (GGG[AG][ACT][CT][CT][ACT]) | 2249-2258 |

CAV replicates via a circular double-stranded replicative intermediate and transcribes an unspliced polycistronic mRNA molecule containing the three partially overlapping genes, each with its own start and stop codons (Noteborn and Koch, 1995). The start of transcription or the 5' terminal of the transcript is suggested to be located at bps 329 to 325 (Phenix *et al.*, 1994) and the end or 3' terminal is probably at bp 2317 (Noteborn *et al.*, 1992a) or at bp 2313 (Phenix *et al.*, 1994). The length of the polycistronic, polyadenylated RNA transcript would be approximately 2.1 kb (Noteborn *et al.*, 1992a).

The overlapping nature of the CAV genes seems to be closely related with the order of CAV protein expressed. Viral protein expression kinetics showed that VP2 and VP3 were preferentially expressed early after the infection and deposited within the nucleus, as early as 12 h onwards; while VP1 is usually observed 30 h post infection (Douglas *et al.* 1995). The early expression of VP2 and VP3 may suggest their relation to viral replicative and transcriptional functions (Douglas *et al.* 1995). VP1, on the other hand, has been associated with CAV viral capsid (Todd *et al.*, 1990; Noteborn *et al.*, 1992a).

## Major CAV Protein Products

### VP1 and VP3 Proteins

As mentioned previously, the three major proteins produced by CAV are the VP1, VP2, and VP3. VP1 has been found to be the only protein component found in the make up of the CAV viral capsid (Todd *et al.*, 1990; Noteborn *et al.*, 1992a). The VP1 protein, of size 52 kDa, with 449 amino acids, is the largest protein produced by CAV. The N-terminal of VP1 protein, which was reported to be highly homologous to protamine (histone proteins) of Japanese quail, cuttlefish, and chicken nuclear protamine, seems to be highly positively charged (Pallister *et al.*, 1994) possibly due to high content of arginine (Claessens *et al.*, 1991), and to have a high affinity for binding and protecting DNA within the virus capsid (Claessens *et al.*, 1991; Meehan *et al.*, 1992; Pallister *et al.*, 1994).

Renshaw *et al.* (1996) suggested another interesting feature pertaining to the role of a hyper variable region, spanning amino acid positions 139 to 151, discovered in VP1 in determining the slow rate of CAV replication or the spread of infection. They also reported, based on amino acid differences in different isolates, that five amino acids of the VP1 upstream from the hyper variable region seem to be involved in permitting or blocking of CAV infection. The relationship between the amino acid differences and the block of infection might involve a conformational change in VP1 or possibly an interaction between VP1 and VP2 through presently unknown mechanisms (Renshaw *et al.*, 1996). Since VP1 is the capsid protein, it becomes the obvious target for vaccine